USP19-deubiquitinating enzyme regulates levels of major myofibrillar proteins in L6 muscle cells

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The ubiquitin-proteasome system plays an important role in the degradation of myofibrillar proteins that occurs in muscle wasting. Many studies have demonstrated the importance of enzymes mediating conjugation of ubiquitin. However, little is known about the role of deubiquitinating enzymes. We previously showed that the USP19-deubiquitinating enzyme is induced in atrophying skeletal muscle (Combaret L, Adegoke OA, Bedard N, Baracos V, Attaix D, Wing SS. Am J Physiol Endocrinol Metab 288: E693–E700, 2005). To further explore the role of USP19, we used small interfering RNA (siRNA) in L6 muscle cells. Lowering USP19 by 70–90% in myotubes resulted in a 20% decrease in the rate of proteolysis and an 18% decrease in the rate of protein synthesis, with no net change in protein content. Despite the decrease in overall synthesis, there were ∼1.5-fold increases in protein levels of myosin heavy chain (MHC), actin, and tropomycin T and a ∼2.5-fold increase in tropomyosin. USP19 depletion also increased MHC and tropomyosin mRNA levels, suggesting that this effect is due to increased transcription. Consistent with this, USP19 depletion increased myogenin protein and mRNA levels approximately twofold. Lowering myogenin using siRNA prevented the increase in MHC and tropomyosin upon USP19 depletion, indicating that myogenin mediated the increase in myofibrillar proteins. Dexamethasone treatment lowered MHC and increased USP19. Depletion of USP19 reversed the dexamethasone suppression of MHC. These studies demonstrate that USP19 modulates transcription of major myofibrillar proteins and indicate that the ubiquitin system not only mediates the increased protein breakdown but is also involved in the decreased protein synthesis in atrophying skeletal muscle.

ubiquitin-specific processing protease

SKELETAL MUSCLE IS THE MOST ABUNDANT TISSUE in the human body, accounting for approximately one-half of body weight. As a major site of metabolic activity, it directly converts chemical energy into mechanical energy and heat. It is also the largest reservoir of protein in the body. During starvation, muscle protein is degraded to provide amino acids for energy and for gluconeogenesis to maintain blood glucose. Muscle protein catabolism also occurs in a variety of pathological conditions such as disuse, loss of innervation, cancer, sepsis, hypercortisolism, and uncontrolled diabetes mellitus and results in significant morbidity (reviewed in Ref. 25). Therefore, much attention has been given to elucidating the biochemical mechanisms involved in mediating protein catabolism in muscle. These studies have shown that ubiquitin-proteasome-dependent proteolysis plays an important role in this process (reviewed in Ref. 1).

The highly conserved 76-amino acid peptide ubiquitin is expressed in all eukaryotic cells and exerts its cellular functions through its ability to be conjugated to other proteins. This conjugation is catalyzed sequentially by a cascade of three enzymes: ubiquitin-activating enzyme (E1), ubiquitin-conjugating enzyme (E2), and ubiquitin protein ligase (E3) (reviewed in Ref. 35). When conjugated as a chain of more than four ubiquitin moieties, it can target the attached protein to the proteasome for degradation (41). Previous studies have shown that, in various catabolic conditions, steady-state levels of ubiquitinated proteins increase in the atrophying muscles (2, 26, 29, 42, 47). This suggests that there is an activation of ubiquitination that increases flux through the ubiquitin-proteasome pathway by increasing availability of ubiquitinated substrates for the proteasome. Supporting this model are many studies that have shown increased expression of many components involved in ubiquitin conjugation in various forms of muscle wasting (reviewed in Ref. 44).

Increased expression in skeletal muscle of polyubiquitin genes (2, 4, 27, 40, 43, 46), ubiquitin-conjugating enzymes E214k, UBC-E2G, and UBC4 (7, 28, 40, 45), and ubiquitin protein ligases atrogin-1 (MAFBx), muscle RING finger-1 (MURF-1) (3, 14), and UBR2 (22) has been described in catabolic conditions. Increased expression of proteasome subunits (2, 5, 28, 36) has also been seen. Solid evidence for a role of the ubiquitin proteolytic system was confirmed with studies demonstrating that genetic inactivation in mice of the E3s MURF-1 or MAFbx/atrogen-1 (3) or of the ubiquitin-binding protein ZNF216 (18) leads to decreased loss of muscle mass following denervation.

Up to now, most of the studies of the ubiquitin system in muscle wasting have focused on the enzymes involved in the conjugation of ubiquitin to proteins. However, ubiquitination can also be modulated by deubiquitinating enzymes (reviewed in Ref. 38), and therefore, these may also be involved in the wasting process (44). Structurally, deubiquitinating enzymes can be divided broadly on the basis of sequence homology into five classes (reviewed in Ref. 33). The largest class (∼75 in the human genome) are the ubiquitin-specific processing proteases (USPs). USPs are recognized by the presence of a core catalytic domain of ∼450 amino acids delimited by cysteine and histidine boxes. Surrounding sequences are more divergent and are likely involved in mediating substrate specificity and other protein interactions. To date, only two deubiquitinating enzymes, USP14 and USP19, have been observed to be regulated in atrophying muscle, and both were induced rather than suppressed, suggesting that they do not act as negative regu-
lators of ubiquitination of the targets of the ligases that are induced in the same catabolic muscles. Two other enzymes, USP2 and USP28, appear to be regulated by muscle stretch (39). USP14 (orthologous to yeast UBP6) was observed to be upregulated in a gene array study of muscle mRNA expression in various conditions of wasting (24). This enzyme is widely expressed and associated with the proteasome (17) and appears to modulate substrate ubiquitination and proteasome activity. We previously identified USP19 as a USP that is induced in skeletal muscle atrophy in response to various catabolic conditions, including cancer, fasting, diabetes, and glucocorticoids (10). The expression of USP19 correlated inversely but very well with muscle mass, consistent with a role in atrophy (10). To further explore the potential functions of this enzyme in muscle atrophy, we determined the effects of siRNA-mediated depletion of this enzyme on the expression of major myofibrillar proteins in L6 myotubes.

MATERIALS AND METHODS

Cell culture and transfection. L6 rat muscle cells (Dr. Amira Klip, University of Toronto) were grown at 37°C with 5% CO₂ in α-MEM (GIBCO) supplemented with 10% fetal bovine serum and 1% penicillin-streptomycin. Cells were induced to differentiate by replacing the medium with α-MEM supplemented with 2% fetal bovine serum and 1% penicillin-streptomycin. L6 cells were seeded in six-well plates at a density of 1.9 × 10⁵ cells/well (for Western blot analysis) or in 60-mm plates at a density of 3.8 × 10⁵ cells/plate (for Northern blot analysis) and incubated overnight in growth medium. The next morning, the cells were ~80% confluent and were transfected with 25 nM USP19 siRNA or nonspecific control (CTL) siRNA oligonucleotides (IDT) using Lipofectamine and Plus Reagent (Invitrogen), following the manufacturer's protocol. For myogenin and USP19 double-siRNA experiments, cells were transfected with 50 nM CTL, 25 nM CTL, and 25 nM USP19 or 25 nM CTL and 25 nM myogenin siRNA oligonucleotides. Three hours posttransfection, the transfection mixture was supplemented with an equal volume of α-MEM containing 20% fetal bovine serum, and the cells were grown overnight at 37°C. Some samples were harvested 24 h posttransfection (day 0), whereas the remaining cells were induced to differentiate into myotubes by incubating the cells in differentiation medium. Samples were harvested at indicated time points up to day 4 of differentiation. The differentiation medium was changed every other day. To induce catabolism in the myotubes, the synthetic glucocorticoid dexamethasone (Sigma-Aldrich) was added to L6 myotubes in differentiation medium on day 2. On day 3 the medium was replaced with fresh medium containing dexamethasone, and the cells were harvested on day 4.

siRNA sequences were as follows: nonspecific CTL, 5′-AAA CUC UAU CUG CAC GCU GAC-3′ and 5′-GUC AGC GUG CAG AUA GAG UUU-3′; USP19 no. 7, 5′-AAG GGU GGU CUU CUA CAG UUG-3′ and 5′-CAU CUG UAG AAC ACC CUA-3′; USP19 no. 43, 5′-5′Phos/GGC GUG ACA AGA AUA AUG ACU UGG T-3′ and 5′-5′Phos/GAC AAG CAG UCA AUG UUC GAC GCA-3′; myogenin, 5′-UAU CCA CUG CAA AUC CUA GUC TTT-3′ and 5′-CAU GGA UUU GUA GAA ATT-3′.

Measurements of rates of protein turnover. Protein synthesis was measured in L6 cells on day 4 of differentiation by incubating the cells for 2 h in differentiation medium containing 5 μCi of [3H]tyrosine/ml and 2.0 mM nonradioactive tyrosine. The amount of labeled amino acid incorporated into protein was determined as described previously (15). To measure protein degradation, muscle cell proteins were radiolabeled after 1 day of differentiation by incubating the cells in differentiation medium containing 1 μCi of [3H]tyrosine/ml and 2.0 mM nonradioactive tyrosine for 48 h. Subsequently, the monolayer of cells was rinsed three times with HBSS containing 2.0 mM nonradioactive tyrosine and then replaced with 2 ml of fresh differentiation medium containing 2.0 mM nonradioactive tyrosine. After incubation for 3 h to allow turnover of proteins with very short half-lives, the process of protein degradation was measured by collecting 0.5 ml of the cell culture medium at various time points ≤33 h and measuring the acid-soluble radioactivity. These subsequent steps were taken as described previously (15).

Western blotting. Cells were lysed by adding 250 μl of 50 mM Tris, pH 7.5, and 2% SDS into the wells of the six-well plate, followed by scraping to collect the cell lysate. DNA in the lysate was sheared using a 1-ml insulin syringe, and the protein concentrations of the samples were determined by the BCA Micro Protein Assay (Thermo Fisher Scientific). Samples (20 or 40 μg) were subjected to SDS-PAGE, and the proteins were transferred onto 0.45-μm nitrocellulose membranes for Western blotting. Membranes containing 20 μg of protein were probed with antibodies to myosin heavy chain (MHC; MF20, Developmental Studies Hybridoma Bank), γ-tubulin (Sigma Aldrich), actin (A4700; Sigma Aldrich), troponin (Sigma Aldrich), or tropomyosin (CH1; Developmental Studies Hybridoma Bank). Membranes with 40 μg of protein were probed for USP19 (30), γ-tubulin, myogenin (FSD; Santa Cruz Biotechnology), or Myf5 (sc-302; Santa Cruz Biotechnology). Horseradish peroxidase-conjugated secondary antibodies and ECL Plus were used to detect all of the bound primary antibodies except for MF20, which was detected using an iodinated secondary antibody and a phosphorimager to avoid signal saturation. Levels of individual proteins were normalized to the level of tubulin on the same blot to control for variations in sample loading and transfer to the membrane.

Northern blotting. RNA was isolated by solubilizing the cells in 4 M guanidinium isothiocyanate followed by phenol-chloroform extraction (6). Northern blots were performed by electrophoresing 10 μg of each RNA sample in 1% agarose gels containing formaldehyde, transferring the RNA onto nylon membranes overnight, and then cross-linking the nuclei acid to the membrane with UV light. Single-stranded 32P-labeled probes complementary to the indicated transcripts were hybridized to the membranes at 65°C overnight and, following washing, exposed to phosphorimager screens for quantitation. Levels of individual mRNA transcripts were normalized to the level of 18S ribosomal RNA on the same blot to control for variations in sample loading and transfer to the membrane. 32P-labeled probes were synthesized by PCR using myogenin, MHC, and tropomyosin cDNA templates corresponding to nucleotide fragments 323–774, 5,375–5,929, and 254–611, respectively. The following reverse primer sequences were used for the PCR: myogenin, 5′-GCA- GAAGTGGTGCGGTCTGACAC-3′; MHC (2X isoform), 5′-GGT- CACTTTCCGTCTGTGA-3′ and tropomyosin 5′-AATGCTTTCA AGGTCTGTA-3′.

RESULTS

Depletion of USP19 modestly decreases the overall rates of protein synthesis and degradation. Since USP19 expression was previously observed to be closely but inversely correlated with muscle mass in rodents (10), we determined the effects of loss of USP19 on rates of protein turnover in L6 myotubes (Fig. 1). L6 myoblasts were transfected with USP19 or a nonspecific control siRNA and induced to differentiate into myotubes for 4 days. As was previously observed in rat fibroblasts (30), the USP19 siRNA oligonucleotides were effective, lowering USP19 levels by 70–90% (Fig. 2A). USP19 siRNA reduced the rate of incorporation of [3H]tyrosine into protein by 18% compared with control siRNA. We also measured the rate of proteolysis as the rate of release of acid-soluble radioactivity from cells that had proteins prelabeled for 48 h with [3H]tyrosine. The rate of proteolysis was 20% lower
Depletion of USP19 can inhibit the loss of MHC induced by dexamethasone. Excess glucocorticoids are well known to activate muscle proteolysis and appear to have a preferential effect on myofibrillar proteins, which, although they are the dominant proteins in differentiated muscle, are minor proteins in myotubes in culture. To test this hypothesis, we determined how USP19 affects the rates of protein synthesis and degradation of major myofibrillar proteins in differentiated muscle. Myogenesis is dependent on a family of myogenic regulatory factors consisting of MyoD, myogenin, Myf5, and Myf6 (Mrf4) (reviewed in Ref. 34). MyoD is not expressed in L6 cells, and Myf6 is expressed only in terminal stages of differentiation that are not recapitulated in the in vitro system (11). Therefore, we tested the effect of depletion of USP19 on myogenin and Myf5, which are expressed in L6 cells (Fig. 4). Transfection of the cells with USP19 siRNA oligonucleotides did not affect Myf5 protein levels. However, myogenin protein levels were approximately twofold higher in cells depleted of USP19 on day 1, and they remained high in these cells until day 4, at which time they returned toward control levels. (Fig. 4, A and B). This was despite persistent low levels of USP19 on day 4 and suggests that other adaptations occur to perhaps limit differentiation to an optimal level of maturation. The overall increase in myogenin expression was associated with a slight but statistically significant increase in the extent of muscle cell fusion (defined as %cells containing 2 or more nuclei): CTL siRNA, 74 ± 1.7%; USP19 siRNA, 86 ± 2.9%. To determine whether the increased myogenin protein levels in USP19-depleted cells were due to an increase in the levels of myogenin transcript, Northern blots were performed on RNA isolated from cells collected from day 0 to day 3 (Fig. 4C). USP19 siRNA resulted in an approximately twofold increase in myogenin mRNA on day 1 compared with CTL-transfected cells, and this persisted to day 3 (Fig. 4D). Taken together, these results indicate that USP19 regulates the expression of the myogenic regulatory factor myogenin.

USP19 regulates the expression of muscle-specific genes in a myogenin-dependent manner. To test whether USP19 depletion increases the expression of myofibrillar proteins through the increased expression of myogenin, we determined the effect of lowering myogenin levels on the ability of USP19 to modulate expression of MHC and tropomyosin (Fig. 5). We selected an siRNA oligonucleotide that would not silence myogenin expression completely but would, upon concomitant treatment with myogenin and USP19 siRNA oligonucleotides, result in myogenin levels similar to levels in control siRNA-transfected cells. As described above, silencing of USP19 increased levels of myogenin (Fig. 5A), MHC (Fig. 5, B and C), and tropomyosin (Fig. 5, B and D). However, these increases were no longer seen when myogenin was simultaneously depleted (Fig. 5, B–D). These results argue that USP19 does indeed act through myogenin to negatively regulate the expression of major muscle proteins.
We observed previously that USP19 expression increases in catabolic conditions, including dexamethasone treatment (10). Other investigators have reported that incubation of L6 myotubes with dexamethasone can increase overall protein breakdown (32) and lower levels of MHC (13). Therefore, we tested whether dexamethasone can induce USP19 in muscle cells and whether lowering USP19 in muscle cells can reverse the loss of MHC induced by this catabolic stimulus (Fig. 6). We observed a small (~20%) but reproducible increase in USP19 levels in the dexamethasone-treated myotubes and confirmed that dexamethasone is able to lower MHC levels in L6 myotubes (Fig. 6, A and C). Silencing of USP19 in these muscle cells was indeed able to reverse the loss of MHC protein induced by dexamethasone to levels similar to cells treated with control siRNA but not to the higher levels seen with USP19 siRNA alone (Fig. 6, A and B).

**DISCUSSION**

We have demonstrated for the first time that a deubiquitinating enzyme can regulate the expression of a panel of major myofibrillar proteins, including MHC, actin, tropomyosin, and troponin. Loss of USP19 in L6 myotubes increased the level of these proteins (Fig. 2). Although it is known that myofibrillar proteins such as MHC, myosin light chains (8, 9), and troponin I (21) are ubiquitinated in muscle, these myofibrillar proteins do not appear to be direct substrates of USP19, because siRNA-mediated loss of this deubiquitinating activity would have increased their ubiquitination and degradation and therefore lowered rather than raised their levels. Instead, our data argue that USP19 negatively regulates a transcriptional program of muscle-specific genes. mRNA levels of MHC and tropomyosin were found to be elevated upon USP19 depletion.
Myogenic regulatory factors are a group of muscle-specific transcription factors that are master regulators of skeletal muscle development (34). We found for the first time that USP19 can modulate transcription of myogenin, a myogenic regulatory factor member that is increased during muscle differentiation, because silencing of USP19 increased levels of both myogenin mRNA and protein (Fig. 4). However, USP19 does not necessarily act at the beginning of the differentiation process, because similar increases in expression of MHC could be observed when L6 cells were transfected with USP19 siRNA oligonucleotides on day 2 of differentiation and harvested on day 4 (data not shown). In addition, the increase in myogenin was associated with only a small, 15% increase in the number of myotubes, suggesting that most of the stimulus of myogenic differentiation is achieved with myogenin levels present in the cells transfected with the control oligonucleotides. The mechanism by which USP19 regulates myogenin remains unclear. The simplest model is that a transcriptional repressor of myogenin transcription is a substrate of USP19 and can be destabilized upon silencing of USP19. However, at this time, we cannot exclude the possibility that USP19 acts on substrate(s) upstream in the signaling pathways that modulate the synthesis of myofibrillar proteins.

Silencing of USP19 did lower the rate of overall protein degradation by 20%, and we cannot exclude that this small change contributes to the overall increase in steady-state levels

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of myofibrillar proteins. However, in view of the previously measured half-life of 27 h for MHC in myotubes (16), USP19 siRNA would have to prolong this half-life markedly to induce a 50% increase in steady-state levels. In addition, the fact that preventing the increase in myogenin expression completely blocks the increase in myofibrillar proteins (Fig. 5) also argues that proteolysis does not play a major role in the regulation of myofibrillar protein levels by USP19.

Fig. 5. USP19 inhibits the expression of muscle-specific genes in a myogenin-dependent manner. L6 myoblasts were transfected either singly with nonspecific CTL, USP19 (no. 7), or myogenin siRNA or doubly with both myogenin and USP19 siRNA oligonucleotides and induced to differentiate for 3 days. Cell lysates were analyzed by Western blotting for myogenin and USP19 (A) and MHC and tropomyosin (B). Effects of single and double siRNA-mediated depletion on tropomyosin and MHC proteins were quantified in C and D, respectively (n = 4). *P < 0.05 compared with CTL (1-way ANOVA).

Fig. 6. Depletion of USP19 can reverse dexamethasone-induced loss of MHC. L6 myoblasts were transfected with nonspecific control (CTL) or USP19 siRNA (no. 7) oligonucleotides. On day 2 of differentiation, the developing myotubes were treated with 10 μM dexamethasone or vehicle. Two days later, the cells were harvested and the lysates analyzed by Western blotting for the indicated proteins. A: representative blots. B and C: quantitation of MHC and USP19 protein levels (n = 3). Shown are means ± SE. *Means that are statistically significant with P < 0.05 (1-way ANOVA).
As mentioned above, a study using gene arrays to identify mRNA transcripts regulated in various conditions of muscle wasting identified USP14 as being induced in wasting conditions (24). This proteasome-associated deubiquitinating enzyme is homologous to yeast UBP6, and genetic manipulation in yeast indicates that it inhibits protein degradation by deubiquitinating substrates and inhibiting proteasome activity (17). Thus, in contrast to our observations on USP19 reported here, the role of induction of this enzyme in conditions of muscle atrophy remains uncertain.

In our previous study, USP19 mRNA levels increased by 30–200% in rat skeletal muscle atrophying in response to cancer, glucocorticoids, fasting, or diabetes. During refeeding, the increased mRNA levels of USP19 in the fasted state returned to normal, coinciding with the recovery of muscle mass (10). Together with our loss of USP19 function in studies in cultured cells reported here, this suggests that USP19 has a specific role in regulating levels of myofibrillar proteins in adult differentiated muscle. Although myogenin levels are induced in adult myofibers upon denervation, studies involving other forms of muscle wasting have reported lower levels of myogenin expression in atrophying muscle (12, 37), suggesting that the mechanism that we have identified in cultured muscle cells may be relevant in vivo.

Steady-state levels of muscle proteins are determined by the balance between their rates of synthesis and degradation. In many catabolic conditions, muscle atrophy arises from both a fall in the rate of synthesis and an activation of the rate of degradation (reviewed in Ref. 19). A large body of previous work has shown that components of the ubiquitin-conjugating apparatus are induced in atrophying muscles, indicating that the ubiquitin-proteasome pathway contributes to the increase in muscle protein degradation (44). A recent report indicates that the induction of the MAFbx ligase may also contribute to inhibition of protein synthesis by targeting the eIF3 initiation factor for degradation (23). To our knowledge, we are showing for the first time evidence for an important role for a deubiquitinating enzyme in muscle atrophy and evidence that this enzyme may contribute to wasting by inhibiting the synthesis of major myofibrillar proteins. Our results thus expand the functions of the ubiquitin system in muscle physiology. Consistent with a potentially important role of USP19 in pathological conditions of muscle wasting, we found that depletion of USP19 reversed the loss of MHC induced by dexamethasone in L6 myotubes and that this catalytic stimulus can induce expression of USP19 (Fig. 6). Transfection with USP19 siRNA in the presence of dexamethasone did not increase MHC levels to levels seen with transfection of USP19 siRNA alone. This suggests that USP19 depletion cannot reverse all of the effects of glucocorticoids on MHC. Indeed, glucocorticoids promote muscle catabolism through multiple mechanisms, including decreased insulin and IGF-I signaling, increased myostatin expression, decreased translation, and activation of transcription factors that lead to induction of ubiquitin protein ligases (reviewed in Ref. 31). Therefore, our results are consistent with USP19 modulating glucocorticoid effects specifically on the transcriptional pathway that leads to synthesis of myofibrillar proteins. At this time, we cannot exclude the possibility that the siRNA and glucocorticoid effects on MHC are independent of each other and additive. Further exploration of this new mechanism of muscle wasting will require the study of USP19-deficient mice. Nonetheless, our present findings suggest that inhibition of USP19 could be a novel therapeutic mechanism for the prevention and treatment of muscle protein catabolism.

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DISCLOSURES

No conflicts of interest are declared by the author(s).

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